Keratinocyte Stem Cells: a Commentary[1]

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For many years it has been widely accepted that stem cells play a crucial role in adult tissue maintenance. The concept that the renewing tissues of the body contain a small subcompartment of self-maintaining stem cells, upon which the entire tissue is dependent, is also now accepted as applicable to all renewing tissues. Gene therapy and tissue engineering are driving considerable interest in the clinical application of such hierarchically organized cellular compartments. Recent initial observations have provided a tantalizing insight into the large pluripotency of these cells. Indeed, scientists are now beginning to talk about the possible totipotency of some adult tissue stem cells. Such work is currently phenomenologic, but analysis of data derived from genomics and proteomics, identifying the crucial control signals involved, will soon provide a further impetus to stem cell biology with far reaching applications. The epidermis with its relatively simple structure, ease of accessibility, and the ability to grow its cells *in vitro* is one obvious target tissue for testing stem cell manipulation theories. It is crucial, however, that the normal keratinocyte stem cell is thoroughly characterized prior to attempting to manipulate its pluripotency. This commentary assesses the data generated to date and critically discusses the conclusions that have been drawn. Our current level of understanding, or lack of understanding, of the keratinocyte stem cell is reviewed. ***Key words: clonogenic cells/ EPU/ hair follicle/ hair follicle bulge/ pluripotency/ skin/ stem cells/ stem cell markers. J Invest Dermatol 119:888–899, 2002***

The concept of stem cells with their dependent cell lineages as applied to epidermis can be traced back around 30 y (Potten, 1974). More recent work based on cell culture studies (Barrandon and Green, 1987; Rochat *et al*, 1994) and a wide range of *in vivo* studies in the mouse (Al-Barwari and Potten, 1976; Morris *et al*, 1986; Cotsarelis *et al*, 1990; Lavker *et al*, 1993; Morris and Potten, 1999) have suggested a somewhat more complex organization and distribution of stem cells in skin, with stem-like cells implicated at specific locations in the interfollicular epidermis, in the upper regions of the outer root sheath of the hair follicle (the so-called bulge region), and in the germinal matrix of growing hair follicles. The interrelationship between these three separate skin stem cell compartments remains obscure, although it can be hypothesized that the bulge region stem cells represent the most potent reserve population of ultimate stem cells. These may be capable of forming many of the skin structures, suggesting a pluripotency as well as a considerable proliferative potential, although in steady state situations they may spend much of their time in a quiescent state. There are clear indications that during the early phases of skin development in young mice some of the epidermal stem cells and lower follicle stem cells originate from the bulge region of the forming hair follicle (Taylor *et al*, 2000).

The idea of pluripotent, or indeed totipotent, proliferating cells in the early embryo is clearly an accepted fact. Such cells not only are capable of forming all the tissues of the body but indeed, if separated early enough, can form an entire embryo (twin formation). They can be isolated from the inner cell mass of blastocysts and grown and expanded in culture as embryonic stem cells. In addition to allowing the development of many transgenic laboratory animals (most notably mice) this research is currently subject to considerable ethical and legal debate, as it also forms the basis of the *ex vivo* development of human embryos and the whole issue of reproductive cloning (the establishment of human embryonic stem cell lines involves the use of excess embryos developed during *in vitro* fertilization procedures). What is ill-understood at present is how, and to what extent, the differentiation options open to the cells derived from embryonic stem cells are progressively restricted during development, and through into the adult. Recent advances suggest that such restrictions may not be irreversible, however, and that adult tissue stem cells may be capable of a wider range of differentiated options than previously thought (see below). Differentiation of human embryonic stem cells cannot yet be precisely controlled in a reproducibly defined manner to give all the possible cell types that these cells are capable of generating *in vivo*. It is also clear that some significant differences exist between human and mouse embryonic stem cells and the reasons for these remain obscure.

A major challenge for stem cell research over the next decade is to identify the genes and hence the proteins (regulatory factors) that

Manuscript received May 23, 2002; accepted for publication May 27, 2002

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Abbreviations: EPU, epidermal proliferative unit; LRC, label-retaining cell.

[1]Stem cells here are defined as presented in Potten and Loeffler (1990): proliferative cells that exhibit self-maintenance and an ability to produce a large number of progeny (divide a large number of times) during the course of which different differentiation events may occur within the progeny. They are cells that are capable of regenerating the tissue, following injury, and during the course of these activities exhibit a flexibility in their self-maintenance probabilities. They are the cells in a tissue that are ultimately responsible for all cell replacement during the lifetime of the animal, i.e., all other cells in the tissue can be traced back to the stem cells.

control the behavior of stem cells (their reproduction and differentiation), particularly adult tissue stem cells, which do not raise the same ethical problems as embryonic stem cells. Amongst the tissues of the body, hematopoietic stem cells clearly possess the greatest pluripotency, and recent work has suggested that these cells may have an even wider differentiation potential than has been documented in the past. For example, there is evidence that hematopoietic stem cells may be capable of differentiating into hepatocytes (Petersen *et al*, 1999; Alison *et al*, 2000; Thiese *et al*, 2000). Bone marrow derived cells (Y chromosome marked in female tissue) can be found in liver, lung, gastrointestinal tissue, epidermis, and hair follicles, expressing local tissue markers, but they do not appear to undergo clonal expansion in these sites unless the tissue is injured (Alison *et al*, 2000; Lagasse *et al*, 2000; Theise *et al*, 2000; anecdotal). Although these observations have major implications it remains to be convincingly demonstrated that such Y chromosome labeled single cells (generally isolated single cells) do not represent infiltrating lymphocytes or macrophages, engulfed apoptotic fragments of blood cells containing a Y chromosome, or even cells subjected to chromosomal transfer via some ill-understood mechanism. Very recent observations indicate that some of these results, which suggest extensive plasticity of bone marrow stem cells using chromosomal markers, result from the fusion of bone marrow cells with host cells in the target tissue (Terada *et al*, 2002; Ying *et al*, 2002). This reopens the whole question of how versatile adult stem cells really are. The possibility thus exists that, as the factors that determine the restriction in pluripotency are identified and manipulated, stem cells, at least in the adult, may be re-instructed to form an even wider range of differentiated tissues. Indeed, a totipotency of bone marrow or other tissue stem cells may become a possibility. A totipotent adult stem cell may therefore be indistinguishable from an embryonic stem cell.

FIVE MAJOR ISSUES FOR STEM CELL BIOLOGISTS

The major problems in stem cell biology at the moment are as follows.

1 With the possible exception of the hematopoietic stem cells, there are few reliable markers that enable these cells to be identified, studied, and isolated, although the ability to identify keratinocyte stem cells by virtue of their size, integrin expression levels, and their DNA segregation properties seems likely (see below).

2 Again, with the possible exception of some early lineage cells in the bone marrow or peripheral blood, we are not able to selectively expand stem cells *in vitro*, although preliminary studies with intestinal cells (Booth *et al*, 1999; Whitehead *et al*, 1999), epidermal keratinocytes (Rheinwald and Green, 1975; Barrandon and Green, 1987; Pellegrini *et al*, 1999; Ronfard *et al*, 2000), cornea (Rama *et al*, 2001), and liver (Susuki *et al*, 2001) suggest that this objective is close to being achieved. The elegant work of Barrandon and collaborators has shown that keratinocytes isolated from human skin give rise to colonies of different appearances, long-term culture survival, and subcloning capacity (Barrandon and Green, 1987). These studies have provided evidence that one of the types of clones, the so-called holoclones, exhibits stem cell characteristics in that the colonies have a long survival time and are capable of generating further holoclones as well as of producing a mature epithelium *in vivo*. The holoclones also give rise to paraclones, which have very limited division potential and exhibit a more differentiated morphology and an inability to subclone. An intermediate form of clones called meroclones exhibit intermediate properties. Holoclone cultures, however, have been used clinically to provide extensive long-term engraftment on burns patients with apparent expansionary growth (Barrandon and Green, 1987; Pellegrini *et al*, 1999; Ronfard *et al*, 2000).

3 Most studies of stem cells *in vivo* or *in vitro* involve the testing of the functional capabilities of these cells under situations of stress, where the behavior and even the number of stem cells and their early progeny may be altered (the biologic version of the Heisenberg uncertainty principle: see Potten and Loeffler, 1990). Furthermore, as the regulatory factors and processes that determine the differentiation events that distinguish a stem cell from its nonstem cell progeny are largely unknown, it is unclear at what point within a cell lineage a cell ceases to be able to function as a stem cell. If the system is stressed and cells are forced to do things not normally expected of them, the ''cut off'' for this ability to function as a stem cell may be altered (see **Fig 1**).

4 Related to the point above is the question of whether stem cells have a specific type of cell division or mitosis. This relates to the issue of whether in steady state stem cells always divide symmetrically and differentiation or positional signals remove half of the daughters produced, or whether they always divide asymmetrically – and to what extent they can change their mode of division. Asymmetric division implies that one daughter (and just one daughter) from each division is immediately re-established as the stem cell, containing the same genetic information as the parent, whereas the other daughter cell is destined for differentiation. This is therefore a functional asymmetry that may or may not also involve a positional asymmetry within the tissue. This has led to semantic complications in distinguishing actual stem cells that perform stem cell function in steady state from potential stem cells, precursor cells, and progenitor cells. These issues are also intimately linked to the question of whether stem cells intrinsically differ from other proliferative cells in the tissue or whether they are functioning as stem cells only because of instructive signals, perhaps derived from the environment. Is a stem cell inherently different from other dividing cells, or is its location in the tissue instructive or permissive for the expression of stem cell function? The use of terms such as progenitors or precursors only tends to confuse the situation: a cell is either a stem cell or it is not.

5 Finally, it remains unclear whether stem cells have a limited or unlimited division potential, and whether or not they deteriorate in terms of numbers or functional competence with age and whether or not telomere length and telomerase levels in stem cells play a role in skin aging. It would appear that the epidermal proliferative unit stem cells probably divide about 140 times in the life of a laboratory mouse. In the murine small intestine the stem cells may divide 1000 times in the life of a mouse (probably 5000–6000 times in humans) (Marshman *et al*, 2002). Thus, they have a very large division potential. Although there are some signs of a deterioration (delay) in the damage response mechanism their numbers are not decreased with age (Martin *et al*, 1998). In contrast, the potent reserve stem cells in the bulge region probably divide infrequently in undamaged skin. Cancer is a disease associated with aging and it is probable that most (or all) cancers represent a disease originating in stem cells as a consequence of the accumulation of a series of genetic errors. It is interesting to speculate about which skin stem cell compartment basal cell carcinomas and squamous cell carcinomas originate from.

CELLULAR HIERARCHIES AND STRUCTURAL ORGANIZATION

These issues are somewhat alleviated if one considers a stem cell derived lineage of the sort shown in **Fig 1**, where the loss of stem cell functional capability (or differentiation) does not occur at the level of the ancestral cell, but one or two generations down the lineage (this being the differentiation event that separates stem cells from nonstem cells). There may be a gradual loss of ''stemness'' until a critical cut-off point occurs as the differentiation event, i.e., a dilution of critical factors below a given threshold resulting in the loss of all stem cell capability, rendering a cell nonclonogenic (or a transit amplifying cell). With such a scheme in undisturbed steady state conditions, only the ancestral cell actually functions as a stem cell, but under stress conditions or conditions where the ancestral stem cell may be killed and deleted, the early lineage cells are capable of expressing their full stem cell functional capabilities (a good example of the uncertainty problem outlined in 3 above).

With such a hierarchical scheme the concept of asymmetric *versus* symmetric stem cell divisions becomes less complex. Clearly

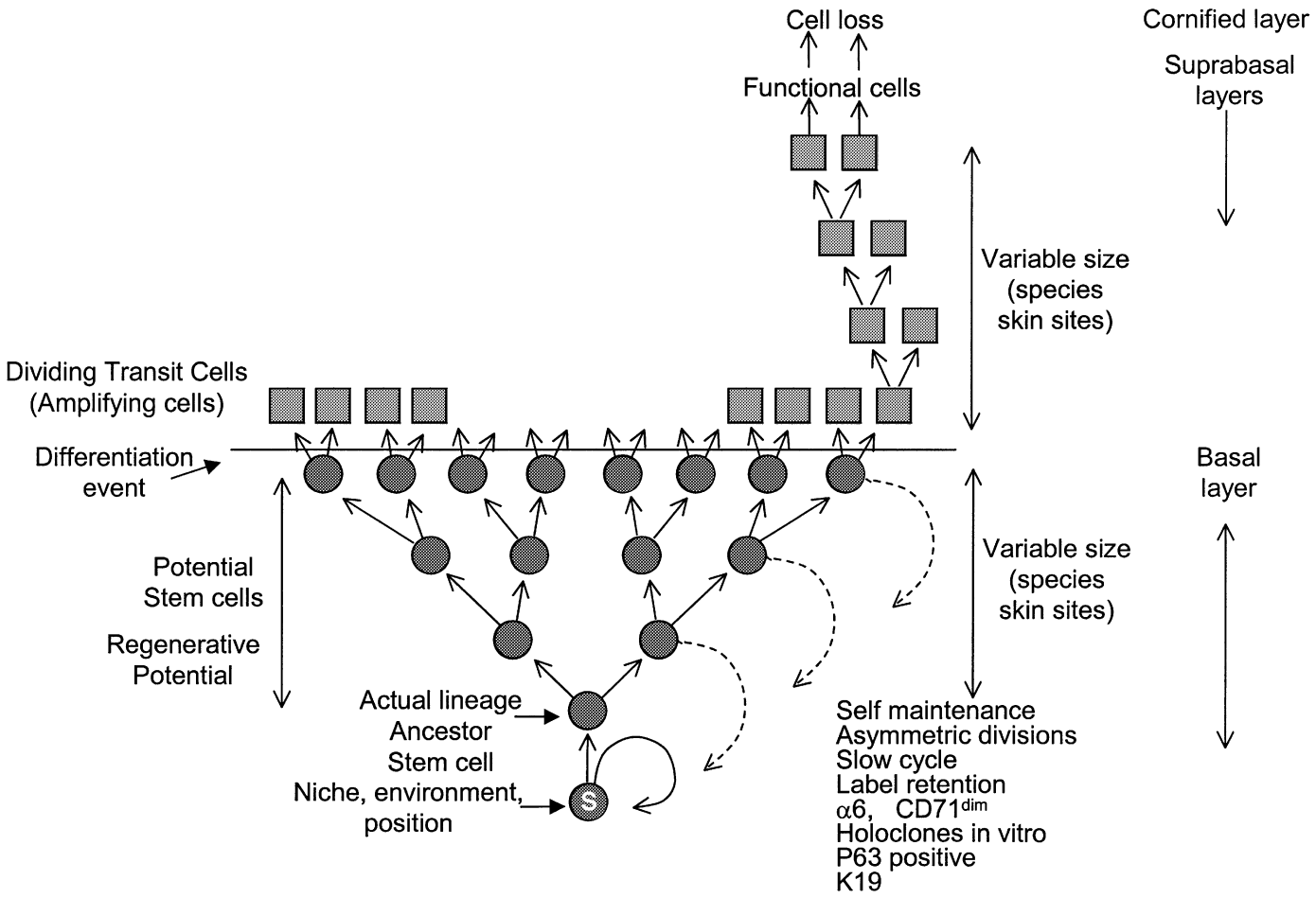

Figure 1. A cell lineage or hierarchy typical of the cell replacement in tissues such as the bone marrow or intestine may also be applicable to the epidermis.

if all stem cells (actual and potential) are considered as indistinguishable, the early cell divisions must be considered as symmetric. Symmetrical divisions would also occur at the point of differentiation. If, however, one considers the hierarchy as a lineage with all of the potential stem cells within a transit part of the lineage, then the initial stem cell division could be thought of as asymmetric, producing transit stem cell daughters that divide symmetrically and eventually differentiate symmetrically, thus ensuring an overall asymmetric scheme for the stem cell compartment. There are some observations that suggest that the ultimate stem cells segregate their DNA in an asymmetric fashion (see below) and hence have a truly asymmetric division process.

One property commonly associated with stem cells is their ability to regenerate the tissue when it is injured (when some stem cells are killed or removed). This has been attributed to these cells because of their large division capacity. The stem cell and subsequent tissue regeneration is achieved by a clonal growth from the surviving isolated stem cells. Stem cells are thus often studied by investigating clonal phenomena and the cells involved are referred to as clonogenic cells (see Potten and Hendry, 1985). It is clear that in some situations steady state stem cells do not equate in terms of numbers or distribution to clonogenic stem cells and hence caution should be exercised in relating these two categories of cells. This is a clear example of the uncertainty principle in operation. Clonogenic stem cells cannot be studied without disturbing the tissue and, in so doing, altering the stem cell status of the tissue.

KERATINOCYTE STEM CELLS

In skin, the epidermis provides the surface barrier, which is enfolded to form various structures including the hair follicle, sebaceous glands, and sweat glands. In terms of stem cell populations it is clear that the interfollicular epidermis is capable of maintaining cell replacement of surface keratinocytes for very long periods of time (even for the full lifetime), providing no localized injury or cell killing occurs. It must therefore have a population of resident stem cells that are the source for all the steady state cell replacement in this stable state.

In the early 1970s it was realized that a considerable degree of histologic organization existed in mouse dorsal epidermis (MacKenzie, 1970; Christophers, 1971; Allen and Potten, 1974; Potten, 1974). The cornified layers consist of thin flat cellular elements (squames), which have a large hexagonal surface area, and these are arranged into columns like a stack of plates. The columns can be traced down to the basal layer within which are a group of about 10 cells with the responsibility for producing cells for that column (to compensate for the surface squames that are constantly being lost). As these structures have stability and a long life, they must be maintained by stem cells. This entire unit was referred to as an epidermal proliferative unit (EPU) and various studies suggested that each EPU contained a single stem cell situated towards the middle of the cluster of 10 basal cells (Potten, 1974; 1981). The EPU were present at about the same frequency as radiation responding clonogenic (regenerative) cells, suggesting that stem and

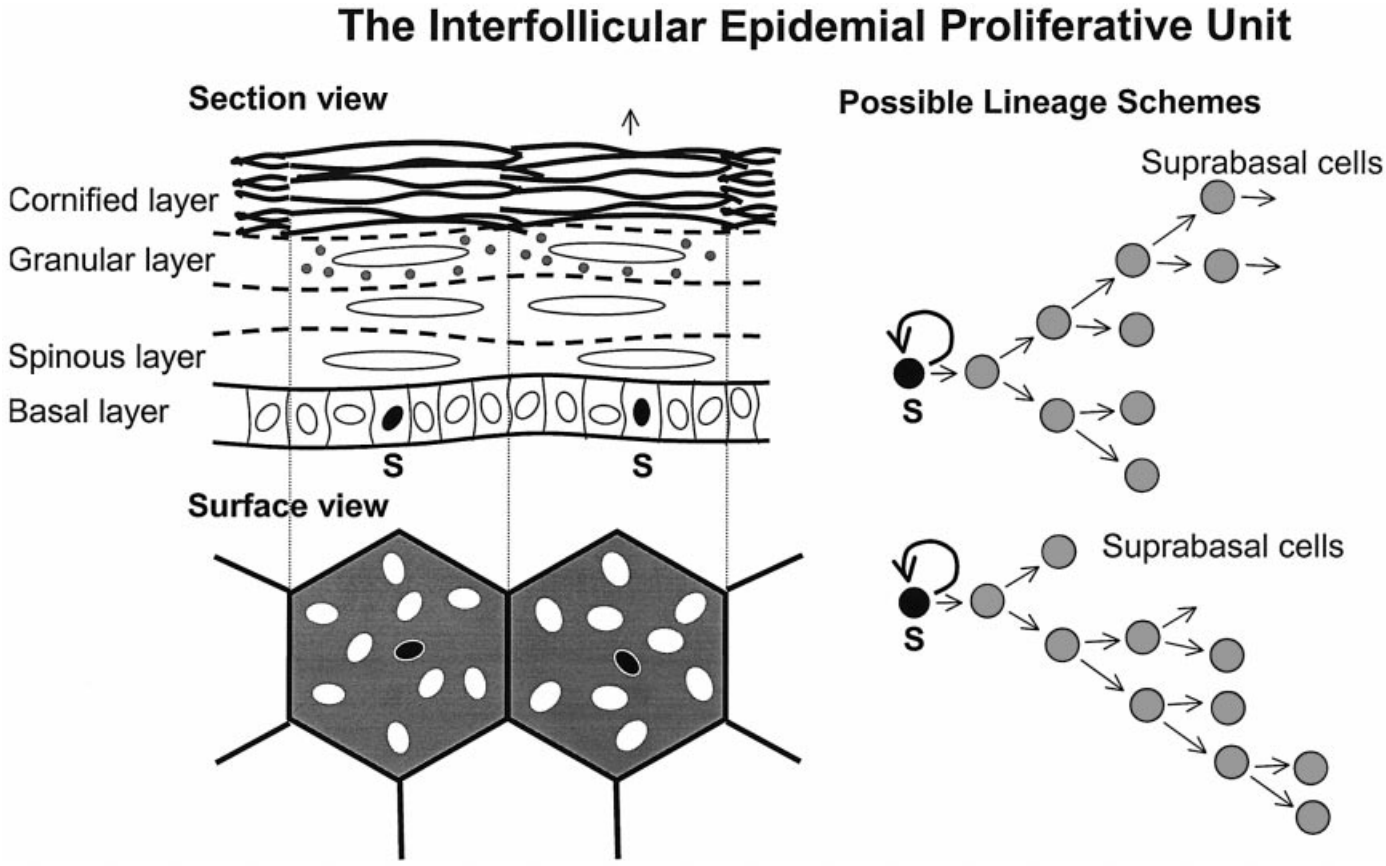

Figure 2. A schematic representation of the murine interfollicular EPUs with their single stem cells and a possible stem-cell-dependent transit lineage. (Modified from Potten, 1974; 1981.) S, stem cells.

clonogenic cells were one and the same (Potten and Hendry, 1973). See **Fig 2**.

Recent experiments where human keratinocytes have been genetically tagged, using retroviral transduction encoding the Lac Z gene, have shown that β-galactosidase expression in epidermis reconstituted by grafting into athymic mice occurred in patches consistent with the concept of the EPU with a stem cell at its origin (MacKenzie, 1997; Kolodka *et al*, 1998).

In the regions of the human body where the epidermis is relatively thin (abdomen, forearms, thigh, buttocks, etc.) with a flat stratum corneum and relatively flat or only mildly undulating basal layer, the structural organization appears to be similar to that of the mouse. The number of strata is greater, but a similar columnar (stacking) organization in the stratum corneum is clearly evident. Although the boundaries of the columns cannot be traced to the basal layer in man in the same way as they can in the mouse, the existence of such columns implies some compartmentalization comparable to the EPU of the mouse (**Fig 3**). The existence of such columns indicates a long-term stability and a controlled process of cell proliferation or cell migration from the basal layer, and by inference a long-term stable organization would be expected to be associated with a stem cell compartment in the basal layer.

Some controversy exists, however, concerning the proliferative organization (and hence stem cell location) in regions of epidermis that are thicker and where there is an undulating basal layer but nevertheless a flat stratum corneum. In these regions the number of strata differs depending on whether a measurement is made at the top of an undulation or at its base. Some reports suggest that stem cells are located at the top of the ridges, whereas others suggest a location at the base of the ridge. From a cell migration viewpoint the latter implies a slightly simpler migratory sequence.

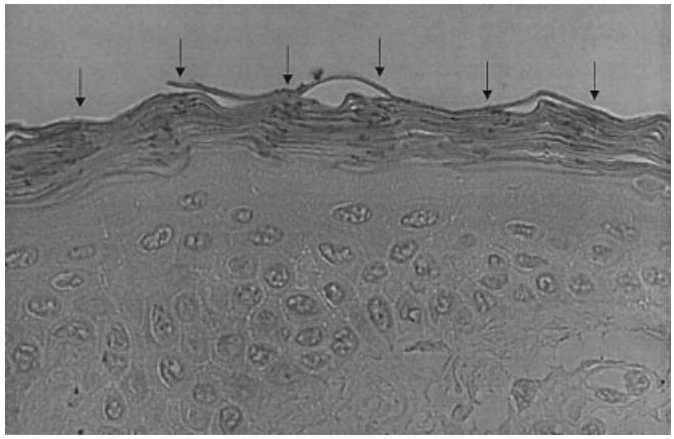

Figure 3. Photomicrograph of human buttock epidermis showing clear EPU-like columns of cornified cells. (From a collaborative study with Professor A. Young.)

The epidermis has evolved as a tough protective outer covering for the body as evidenced by the extensive number of desmosomes and the complex interdigitation of cytoplasmic membrane folds seen particularly in the spinous layer. Such observations suggest that these cells are not a fluid interchanging and mobile set of cells, like soap bubbles, but have evolved mechanisms to restrict cell movement and hence reduce epidermal weaknesses. The well-organized structure suggests a coordinated movement of cells, both along the basal layer from the deepest regions to the shallowest

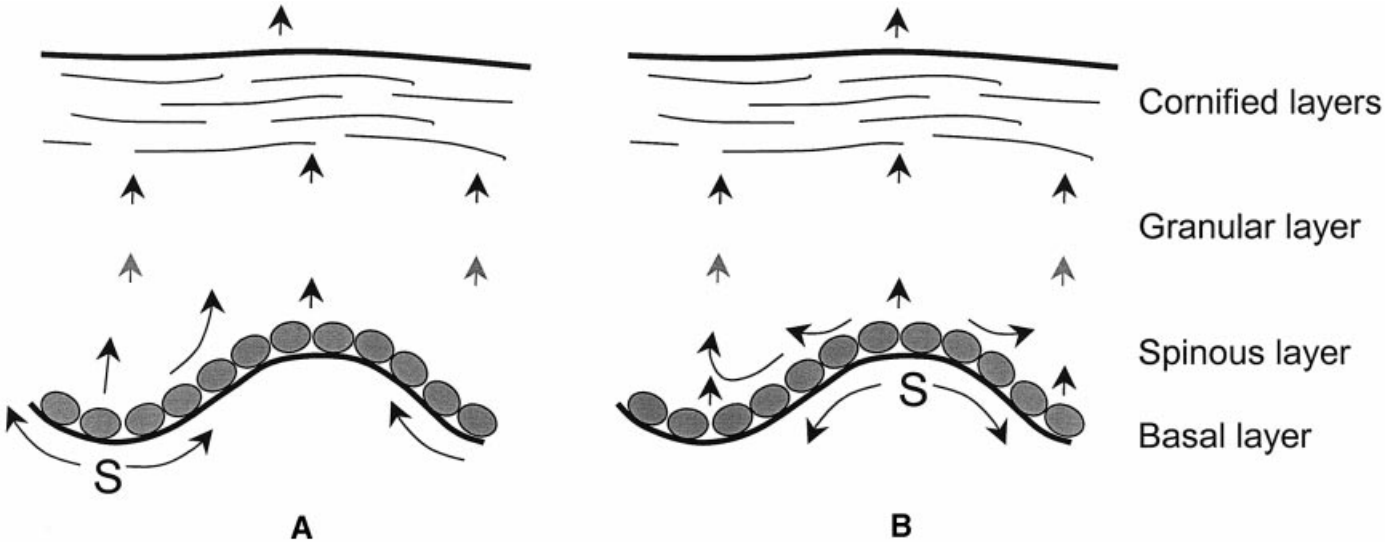

Figure 4. Schematic diagram of human epidermis with a rete ridge (or peg) structure. The arrows show cell migratory pathways. (*A*) Stem cells are located at the deepest point in the epidermis, at the base of the epidermal ridge or peg. (*B*) Stem cells are located at the shallowest point in the epidermis. Here slightly more complex cell migratory patterns are required.

ones, and through the strata whether it be the thick or the thin regions in a rete ridge (or peg) type of organization. In such a kinetically stable structure, one would not expect situations to exist where movements of cells occurred in conflicting directions. Unless cells can overtake each other in their outward migration, which seems highly unlikely bearing in mind the strong functional binding and the requirement for strength, the migration from (and hence cell production along) the basal layer must be tightly regulated to ensure a coordinated movement of strongly bound cells at both the thin and the thick regions. When a cell migrates into the spinous layer at the thinnest region it might bind (via desmosomes) to lateral neighbors that migrated some time earlier in the thickest regions. Either model in **Fig 4** could operate; however, model (A) is somewhat simpler and is favored by many workers in the field. There are also some indications that the cells at the base of the rete ridge (deepest epidermal points) are less differentiated in appearance than cells at the tip of the rete ridge (serrated cells) (Lavker and Sun, 1982; 1983). Model (B) also has its advocates, however (Jones *et al*, 1995; Jensen *et al*, 1999) (**Fig 4**).

STEM CELL CYCLE DURATION

A great deal of weight is apportioned to the fact that adult tissue stem cells generally have a long cell cycle time or spend much of their time out of cycle (in G_0 or quiescence) and as a consequence it is often stated that this is a property of stem cells. There is no *a priori* reason, however, why stem cells would have to cycle slowly and it may just be coincidental that for most systems (the dorsal surface of the tongue seems to be the exception) stem cells spend long periods of time in G_0/G_1. The advantages of this may be that it allows time for genetic housekeeping and repair of random damage. The existence of a cell lineage or hierarchy also ensures that stem cells need to divide less frequently than tissue replacement requires as much of the cell production is achieved within the stem cell amplifying transit compartment. In mouse skin mathematical modeling of cell kinetic experiments has suggested a stem cell cycle time of about 200 h (approximately 8 d), in comparison with a cycle time of about 100 h for the dividing transit population (Potten *et al*, 1982; Potten and Loeffler, 1987). It is surprising that in skin and gut the stem cells appear to be cycling more slowly than the transit cells by a factor of precisely 2. In the bulge region of the hair follicle the indications are that the cells are even more quiescent. In the germinal region of the follicle, the cycle time of the hair follicle stem cells is unknown but likely to be considerably shorter than 200 h. The transit cell population in the mouse anagen (growing) hair follicle has a cell cycle time of about 12 h. In the human epidermis, detailed information is largely lacking but the stem cell cycle time is likely to be greater than 500 h (about 21 d).

Even though the stem cell cycle times may be significantly longer than those of the dividing transit population, it is clear that one property of stem cells is that their cycle times can be dramatically reduced following injury to the tissue or growth *in vitro*. *In vivo* the mouse stem cell cycle times are capable of reductions from 200 h to significantly less than 12 h under wounding conditions. When the hierarchy is re-established, however, the longer cell cycle time is resumed.

HAIR FOLLICLE STEM CELLS

Adult hair follicles go through cyclic phases of very active proliferation (anagen) and hair production and periods of quiescence (telogen) with no proliferation when the follicles contain a mature hair. In the mouse these cycles of growth last precisely 21 d, and therefore waves of hair growth occur at specific times in the life of a mouse (see review by Hardy, 1992). In adult human scalp, each hair follicle tends to behave more autonomously, but still has cycles of growth and dormancy. The cycles of growth here can last for months. In some animal species, like Angora rabbits or Merino sheep, hair growth is almost continuous. When the follicle is growing there is an enormous amount of cell proliferative activity (short cell cycles) in the germinal or matrix region at the base of the follicle. There is a strict spatial organization and migration pathway in this region, suggesting that the ancestral cells for any cell lineage would be located in the very lowest regions of the follicle matrix (the germinal region of the hair bulb), i.e., at the origin of these migratory pathways. The large amount of cell production, sometimes for very long periods of time, would suggest that the growing follicle must contain some cells that sustain this prolonged cell division activity and hence function as stem cells. These putative stem cells would be greatly outnumbered by rapidly dividing transit cells.

UPPER OUTER ROOT SHEATH STEM CELLS (BULGE REGION)

Early studies in the 1960s (Albert *et al*, 1967a; 1967b), radiation studies in the 1970s (Al-Barwari and Potten, 1976), and

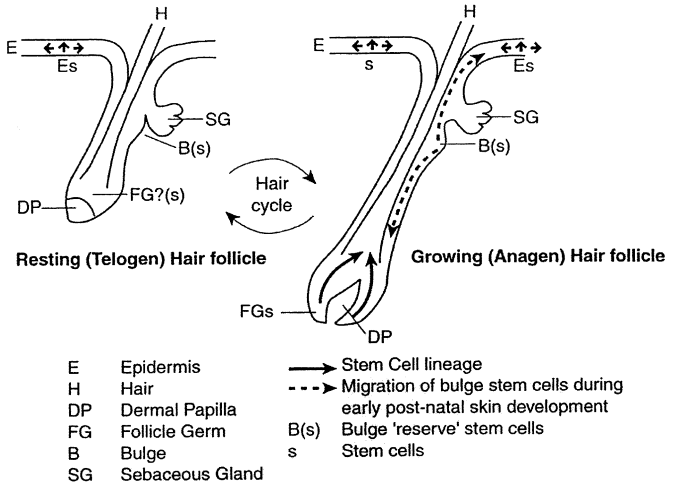

Figure 5. Schematic diagram showing interfollicular epidermis with a telogen (resting) hair follicle (*left*) and an anagen (growing) follicle (*right*) with a summary of current hypotheses on the location of stem cells (S).

dermabrasion studies in the 1980s (Morris and Argyris, 1983) have indicated that the upper regions of the hair follicle also appear to contain potent stem cells, with a regenerative capacity for the epidermis as well as for the hair follicles (and probably also the sebaceous glands). This was supported by an important paper by Cotsarelis *et al* (1990) and Taylor *et al* (2000), who recently demonstrated that, at least in the early phases of skin development, both epidermis and follicular stem cells can be tracked back to a specialized region in the upper outer root sheath of the follicle called the bulge (see **Fig 5**). The role of this regenerative reserve of stem cells in the normal adult hair cycle is less clear, although some data suggest that the matrix is repopulated at each cycle by stem cells from the bulge region (Oshima *et al*, 2001). There are alternative explanations, however (Reynolds and Jahoda, 1991; Morris and Potten, 1999; Panteleyer *et al*, 2001). If the bulge region contributed to new cycles of hair growth one might expect to see a progressive movement of stem cells from the bulge region to the follicle germ, as the anagen growth phase developed or shortly before it developed. In fact the distributions along the axis of the follicle seem to show an increased number of clonogenic cells at the base of the follicle towards the end of the growth cycle (Oshima *et al*, 2001). The authors relate this to the time that it takes for stem cells to migrate over the 2 mm length of the follicle. It seems equally possible that these numbers reflect the practical difficulties of clonal regeneration assays with populations being assessed containing a varying mixture of rapidly dividing transit cells and stem cells.

The question of how stem cells function and are replaced in the growing hair follicle and the role that stem cells in the bulge region of the follicle play in hair production remains somewhat confused. Part of the confusion derives from the fact that some studies investigate the developmental stages of the hair follicle, whereas others use specialized follicles, such as the vibrissae, and yet others use complex tissue injury and grafting techniques. It is unclear to what extent the studies on these systems are applicable to murine pelage hair or indeed to human scalp or body hair. What is fairly clear and generally accepted is that the bulge region of the follicle that is in the upper outer root sheath, or the upper outer root sheath itself where a bulge is not clearly discernible, contains some fairly potent stem cells capable of re-epithelializing the surface epidermis, contributing cells to the forming follicle during skin development, and regenerating the follicle, sebaceous glands, and epidermis following injury. It is evident from various studies, most notably Rheinwald and Green (1975), Kobayashi *et al* (1993), and the recent papers by Taylor *et al* (2000) and Oshima *et al* (2001), that considerable further insight into the role of bulge stem cells in follicle hair cycles has been achieved. The studies also raise a number of further questions, however. Using *in vitro* clonogenicity assays, these authors demonstrated that the bulge region of the growing vibrissal follicle of the rat contains a large number of clonogenic keratinocytes (95% of all those present in the anagen vibrissal follicle), whereas the bulb region contains only a few (5%). The bulge region contains essentially noncycling cells, whereas the bulb is rapidly proliferating. Assuming hierarchical organization in the bulb, however, only a small proportion of the large number of proliferating cells would be expected to be clonogenic stem cells. This observation therefore need not indicate an all-or-none effect for the distribution of stem cells in these two regions, but differing proportions. In further experiments, Barrandon and colleagues (Oshima *et al*, 2001) dissected fragments of the bulge region from mouse vibrissal follicles that were constitutively expressing a Lac Z reporter gene under the control of an SV40 promoter (Rosa 26 transgenics). These bulge regions were transplanted onto the back of wild-type mouse embryos and into vibrissae follicles. These studies showed that when stained for β-galactosidase the typical blue staining associated with the Rosa 26 transgenics was gradually observed throughout the grafted follicle. This process took times in excess of a hair growth cycle for the vibrissae follicle and clearly indicate that, within the framework of these grafting experiments, cells from the bulge can repopulate the entire follicle, including the bulge region of an anagen follicle. Furthermore, these studies showed that the bulge region cells can contribute to all elements of the epidermis and sebaceous glands. It remains somewhat uncertain as to whether the injury caused by the transplant procedure results in the initiation of the follicle regeneration process. It is possible that under normal circumstances the bulge region cells may not constantly supply the follicle matrix with new hair bulb stem cells. Indeed in the mouse pelage follicles, where there is no obvious hair bulge region, similar results could not be obtained. Consistent with the latter observation there are studies suggesting that the label-retaining cells (see below) in the upper follicle outer root sheath do not migrate to the anagen follicle bulb region (Morris and Potten, 1999). In yet another complex grafting experiment, Oshima *et al* (2001) took the Rosa 26 vibrissae follicles growing as grafts on mouse embryos and subsequently transferred them to the back skin of adult athymic mice. This resulted in many β-galactosidase-positive follicles, in this case resembling pelage hair follicles rather than vibrissae. A more informative experiment involved dissecting the Rosa 26 vibrissae follicles into five regions along their length. These fragments were implanted individually onto the back of embryos or young animals and the subsequent development of β-galactosidase-stained follicles was determined from this *in vivo* clonal regeneration type of experiment. Interestingly, follicles did not develop from anagen follicles except from those fragments that contained the upper outer root sheath regions. The anagen follicle bulbs did not appear to contain sufficient numbers of stem cells to ensure a follicular repopulation. Interestingly, the bulb regions from follicles in catagen or early anagen did appear to contain sufficient concentrations of stem cells to effect follicular regeneration. This study was done in conjunction with an *in vitro* clonal assessment, the results of which suggested that the bulge region contained large numbers of clonogenic keratinocytes but the bulb region of the follicle contained a variable, much smaller number, and the number depended on the stage of the hair growth cycle. The greatest numbers were observed in the bulb at the end of anagen and during catagen. The data suggest that the bulb region stem cells are present in numbers that are in proportion to the total number of proliferating cells in the germinal region of the follicle.

It remains uncertain as to whether studies into stem cell behavior in developing pelage follicles, or these complex grafting experiments of segments from adult vibrissae follicle, provide information about the stem cell organization in adult mouse pelage hair growth cycles. It is also a matter of debate as to which, if any, of these models are relevant for human scalp or body hair follicles. This is

another clear example of where caution is needed in the interpretation because of the uncertainty principle. These studies involve considerable disruption of the tissue and as a consequence the stem cells and their behavior are almost certainly altered. At present it is clear that the bulge region contains very potent regenerative stem cells that are normally quiescent and, hence, presumably represent a reserve population of cells. It is equally clear that during the early stages of follicular formation, cells from the bulge contribute to follicle formation. Equally it is true that cells from the bulge region have a potent capacity for follicle regeneration following injury and this may be what is being studied in the various grafting experiments. Many of the observations are consistent with the concept that the pelage follicles contain a relatively constant number of stem cells throughout the hair cycle. The proportion of these relative to the proliferating cells may vary dramatically, however, as the total cellularity in a growing pelage follicle is 2 orders of magnitude greater than that of a resting follicle. Neither the bulge activation hypothesis by the follicular dermal papillae nor the stem cell trafficking hypothesis seem to adequately explain all the observations.

Rochat *et al* (1994) instigated a series of related *in vitro* studies where growing human scalp hair follicles were cut into five segments and the colony forming efficiency of cells within each of these segments was determined. The greatest number of colony forming cells was observed in a segment of the follicle below the arrector pili muscle, i.e., lower than the bulge region but well away from the germinal region at the base of the follicle. The base of the follicle in three separate experiments contained 0.5%, 0.8%, and 1.7% of the colony forming cells, in contrast to 45.3%, 17.7%, and 26.7% for this upper segment. Interestingly, although the number of colony forming cells at the base of the follicle was low, their growth potential *in vitro* was as good as those obtained from higher up the follicle. They divided with the same frequency (cycle time of about 25–26 h) and were capable of the same number of generations (about 120). Thus, although most of the potent clonogenic cells were not located in the germinal region of the growing hair follicle, this region did contain some potent clonogenic cells.

Reynolds and Jahoda (1991) also undertook experiments where the germinal regions of vibrissae follicles were removed and placed *in vitro* in a medium normally conducive to keratinocyte proliferation. Although these cells remained quiescent, small and tightly packed, if cultured in the presence of dermal papillae cells they proliferated rapidly and were able to form follicle-like structures. The authors concluded that the germinal region of the follicle contains stem-cell-like entities, undifferentiated in appearance, small in size, with few organelles but many ribosomes. They were present in low numbers and under appropriate stimulation *in vitro* were capable of extended proliferation and development of rudimentary morphogenic structures. The same authors have also suggested that the follicle is surrounded by dermal progenitor cells that have a myofibroblast phenotype and play an important role in maintaining and regenerating the follicle dermal papilla (Jahoda and Reynolds, 2001). This may have significance during wound healing and in determining the levels of scar formation.

Based on carefully timed labeling studies and a review of literature dating back more than 50 y, Panteleyer *et al* (2001) have suggested that the catagen follicle contains a bulge-derived cluster of cells referred to as the lateral disc, which is incorporated into the telogen hair germ. This group of cells then contributes to the formation of the next anagen hair follicle germinal matrix. This concept, termed hair follicle predetermination, provides an interesting compromise between the two opposing concepts for hair follicle renewal, as it incorporates elements of both hypotheses.

The S100 family of calcium binding proteins vary in expression throughout the hair cycle, with S100A4 being expressed in the bulge region and S100A6 in the anagen follicle bulb (Ito and Kizawa, 2001). These proteins may play important roles in the activation of hair follicle stem cells.

ARE STEM CELLS ANCHORED OR MIGRATORY?

One feature thought by many to be important for stem cells is that they are anchored or firmly adhered to an important microenvironment or niche or located in a protected site – protected from accidental dislodgement into a migrating stream of cells and also protected from the external environment. The idea that stem cells migrate at each hair cycle from a bulge or outer root sheath region to the germinal matrix is at odds with this concept of fixed stem cells. The solution to the problem by the proponents of such migration processes is that it is the immediate daughters of stem cells, and not the stem cells themselves, i.e. the very early dividing transit compartment, that migrate. This implies that the cells in the bulge region should divide to produce these migrating daughters. The bulge region is a very quiescent structure, however. The idea of cells migrating through a stratified epithelium rich in desmosomes and cytoplasmic interdigitations is somewhat counterintuitive. It is known that some cells, such as Langerhans cells, migrate through these tissues but these may be an exception (they are certainly very specialized cells). The concept requires that stem cells, or their immediate daughters, migrate in a unidirectional controlled fashion beneath a tightly bound spinous layer, weaving in between resident outer root sheath cells, over considerable distances, without diversion, disorientation, or mistakes in terms of local or long distance signaling. Occam's razor comes to mind, which states that the fewest possible assumptions are to be made in explaining a thing (Occam, fourteenth century English philosopher), and the simplest explanation for hair follicle stem cells is that the follicle germ contains its own stem cells that pass through phases of quiescence and activity during which their proportional numbers vary. A further problem to address in terms of the stem cell migration theory is how the stem cells know where to go and how they are prevented from acting as outer root sheath epithelial stem cells *en route*. If the follicle is damaged in any way it certainly seems likely that it is repopulated by stem cells from this bulge region.

LABEL-RETAINING STUDIES FOR STEM CELLS

Further insights into stem cell locations within the hair follicle and interfollicular epidermis have come from DNA marking studies. Such studies are based upon the empirical observation that DNA precursors, which are incorporated into epidermal cells during skin development, persist for long periods of time. It was assumed that this label was persisting in the stem cells because these cells cycle more slowly than other members of their dependent lineage, i.e., the incorporated precursors dilute more slowly (see also below). As a consequence, at an appropriate time postlabeling, cells retaining the DNA precursors (markers) can be observed as label-retaining cells (LRCs).

Such experimental LRC approaches clearly work (Bickenbach, 1981; Morris *et al*, 1985; Bickenbach *et al*, 1986). LRCs can be seen scattered in the interfollicular epidermis where their location in the mouse is consistent with other experimental studies on the location of stem cells (Potten *et al*, 1982; Morris *et al*, 1985; Morris and Potten, 1994). These cells also retain radioactively marked chemical carcinogens for long periods of time (Morris *et al*, 1986). LRCs are also seen in the upper regions of the outer sheath of hair follicles (Potten and Morris, 1988; Cotsarelis *et al*, 1990; Morris and Potten, 1999). The retention of label in putative stem cells in the bulge region is not, perhaps, surprising if these cells rarely undergo cell cycle progression and division once the follicle is formed. It is somewhat surprising, however, that label retention persists in the interfollicular epidermis for many weeks, i.e., over a time course when stem cells would have been expected to have divided many times, and hence to have diluted their incorporated label. As stated previously, the best estimate for the stem cell cycle time in adult mouse interfollicular epidermis is about 8 d. Thus, in an 8-wk-old adult, the age at which LRCs are typically visualized (and many LRC studies use older mice, e.g., 11 wk old), the stem cells would have divided at least seven times. As the cell cycle in juvenile and developing epidermis is likely to be much shorter, the number of

stem cell divisions is likely to be 10 or more. Grain dilution by division would normally be expected to reduce the labeling to subthreshold levels after four or five divisions. Hence a slow cell cycle is unlikely to be the sole explanation for the presence of LRCs. It is somewhat surprising that this issue has not been more closely investigated by those working in the field.

The selective DNA strand segregation hypothesis suggested for stem cells by John Cairns in (1975) could account for the persistence of label (see also Potten *et al*, 1978). This controversial hypothesis suggested that stem cells would have evolved various protective mechanisms against genetic damage. DNA replication is an error-susceptible process and a simple way to protect against DNA-replication-induced errors is to sort the old (template) from the new DNA strands and keep the old template strands in the stem cell line. This phenomenon would suggest that label given at a time when new stem cells are being made, during development or tissue regeneration after wounding, would be incorporated into the permanent template DNA strands in the stem cells, which as a consequence would become permanently labeled, i.e., become LRCs, providing the label was made available at the time of the penultimate stem cell amplifying division. The newly synthesized strands (with any replication-associated errors) would always be passed to the daughter destined for differentiation and loss once the steady state situation was reached. Such DNA segregation would be an extremely effective protective mechanism for a tissue, as any replication-induced errors are shed from the tissue within a few days. Recent double labeling studies in the mouse small intestine suggest that the ultimate stem cells in the intestinal cell lineage do indeed selectively sort their DNA strands at mitosis in this way and retain in the stem cell daughter the replication-error-free template DNA strands (Potten *et al*, 2002). The difficulty with such studies is knowing precisely when, during development, to give the label to achieve maximum labeling of LRCs (too early and the label would be diluted, too late and the template strands would not be labeled). Hence, a certain amount of stem cell biologic knowledge is required before the technique can be most effectively employed. The optimum labeling protocol to generate LRCs would be at the penultimate stem cell expansionary divisions. In the intestine this optimum time for LRC generation is quite late in gut development (6 wk post-natum) (Potten *et al*, 2002). The number of LRCs seen, particularly in the epidermis, should perhaps not be over-interpreted, as detailed studies have not been undertaken to determine precisely the optimum time of labeling to maximize the number of LRCs seen at a later stage in the adult.

Double DNA labeling approaches (using BrdU and ^{3}HTdR to determine whether LRCs remain cycling) have also been used in skin as these approaches provide considerably more information. Caution should be employed, however, when bromodeoxyuridine is used for anything other than pulse labeling, as bromodeoxyuridine is teratogenic, is excised by some cells, and may be inherently more damaging to cells than tritiated thymidine. It is also incorporated with a greatly reduced efficiency compared with tritiated thymidine.

STEM CELL MARKERS

Work by Michele DeLuca and colleagues has recently identified a marker that appears to have specificity for keratinocyte stem cells. The p63 transcription factor belongs to a family that includes structurally related proteins such as p53 and p73. Using keratinocyte cultures that generate holoclones and meroclones, it was found that p63 is abundantly expressed in the holoclones and greatly reduced in the meroclones. Interestingly, p63 plays an important role in morphogenesis and the expression pattern in the cultures, with p63-positive cells flanked by cells with little or no p63, which is a pattern that is strongly reminiscent of the EPU organization (Pellegrini *et al*, 2001).

In addition to nuclear markers various attempts have been made in recent years to identify cell surface keratinocyte stem cell markers that can be used to extract the cells for further study *in vitro*. Cell surface adhesion molecules of the integrin family have proved to be particularly interesting. One property assumed for stem cells is that they are less motile or more tightly bound to the basement lamina than transit amplifying cells or postmitotic cells. Jones and Watt demonstrated that the adherent cells with their high colony forming efficiency had the lowest involucrin staining characteristics (Jones and Watt, 1993). The authors suggested that stem cells express high levels of $\alpha_2\beta_1$ integrin (a receptor for collagen and laminins), $\alpha_3\beta_1$ integrin (a receptor for laminins and epiligrin), and $\alpha_5\beta_1$ integrin (a receptor for fibronectin). Two of these, $\alpha_2\beta_1$ and $\alpha_3\beta_1$, are highly expressed in cells that exhibit a patched distribution in human epidermis from various sites of the body. The somewhat confusing aspect of the distribution of the α_3 subunit was that in some sites of the body the strongest expression occurred at the deepest point in the rete ridge (e.g., palm) whereas in other regions of epidermis the positive cells were located at the shallowest point or top of the rete ridge, e.g., scalp. Also, intuitively, the size of the patch of bright cells is too large for it to be purely marking stem cells. This may be a reflection of the fact that this is a marker for not only stem cells but also early lineage precursors, i.e., the potential stem cells (see **Fig 1**). It remains a major question as to why the integrin α_3 positive cells show different spatial expression patterns in terms of the rete ridges in different regions of the body.

The paper by Jones and Watt (1993) also showed that the cells with the highest α_2 integrin receptor density had the highest colony forming efficiency and this was not related to cell size. The work of both Barrandon and Morris has suggested that the stem cells have the smallest size amongst the keratinocyte population (Barrandon and Green, 1985; Morris *et al*, 1991).

It has been proposed (Michel *et al*, 1996) that keratinocyte stem cells exhibit a characteristic keratin cytoskeletal profile. These intermediate filaments are characteristic of epithelial cell populations and exist in the cytoplasm in characteristic patterns of paired basic and acidic molecules. In the epidermis, typically keratins 5 and 14 are expressed in the basal layer, with keratins 1 and 10 being found in the suprabasal layer. The hair follicles can express these keratins but also keratins 6, 16, and 17. Keratin 19 is expressed at low levels in the skin. It is expressed in the bulge region (Lane *et al*, 1991), where it colocalizes with the LRCs. It is not expressed in interfollicular epidermis, but in thicker epidermis it is seen in the deep epidermal rete ridges where it may also coexpress $\alpha_3\beta_1$ integrins. There was more keratin 19 expression in newborn than older foreskin and this correlated with keratinocyte culture lifespan. These observations all suggested that keratin 19 is a valuable marker, or comarker, for cells early in the keratinocyte cell lineage, i.e., stem cells (Michel *et al*, 1996).

These observations on integrin staining patterns raise an interesting question concerning the theoretical size of the stem cell patch as seen in such sections. If a patch contained a number of contiguous stem cells on the basal layer, the cells at the center of the patch might never need to divide. The generation of dividing transit cells could be achieved solely by the stem cells at the edges of the patches. If stem cells nearer the center of the patch divided to produce dividing transit cells, however, the discrete nature of the patch would quickly disappear. Alternatively, if these central cells divided to produce more stem cells, the patch might continuously expand. If the cells at the center in fact do not divide, this should be clearly demonstrable by using appropriate proliferation-associated markers and would indicate that the skin has far more stem cells than is really needed. Nature is rarely inefficient in this way. This pattern of potential stem cells would seem to be unnecessary compared to a situation where there are occasional isolated stem cells distributed perhaps as a line along the tip, or more probably the base, of the rete ridge, these cells dividing in an inherently asymmetric fashion under normal circumstances, generating dividing transit cells or perhaps some potential stem cells (see **Fig 1**) before producing the dividing transit cells that continue proliferating through a lineage. One of the theoretical advantages of a stem cell–transit cell lineage is that the lineage ancestor cells would be a

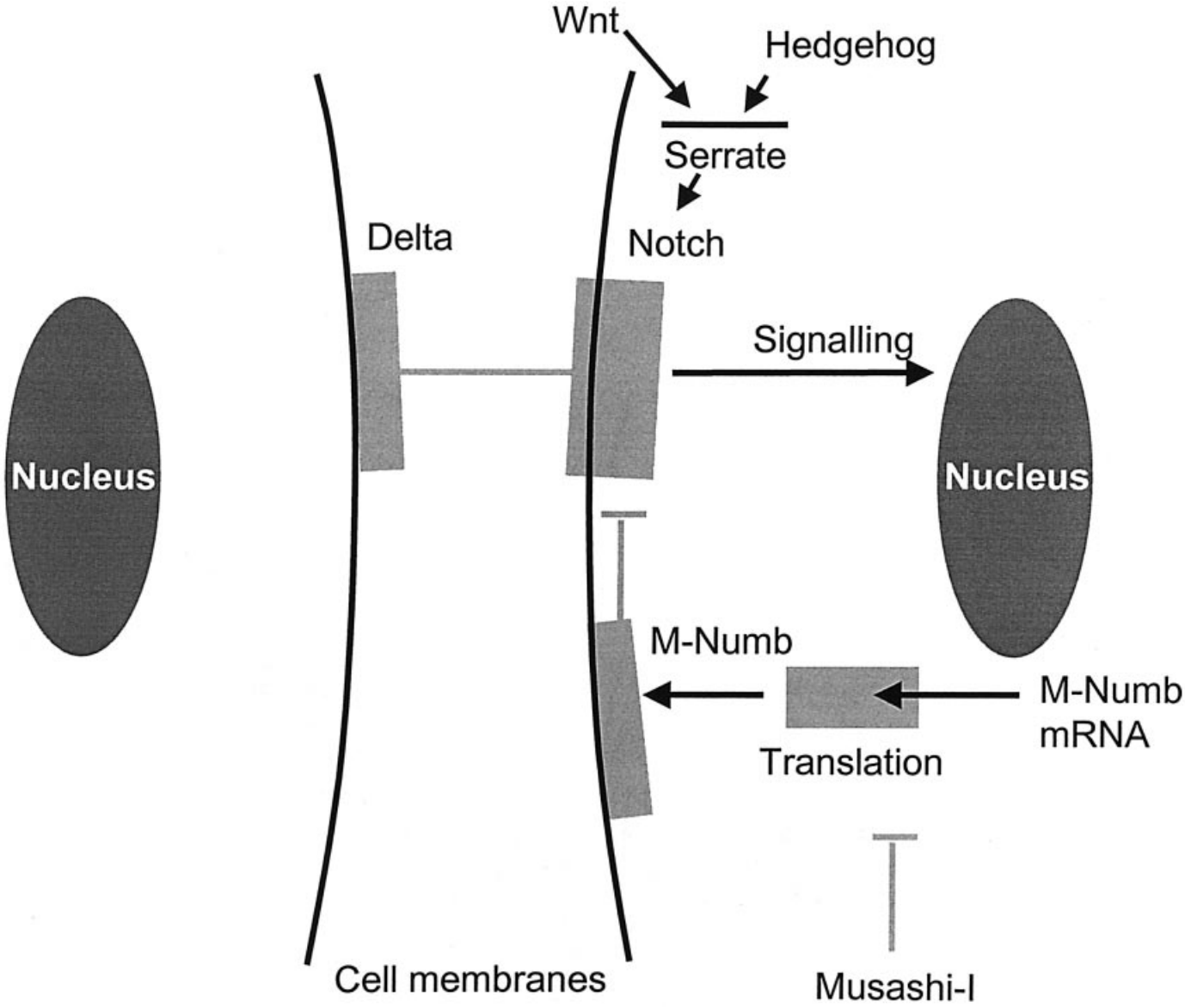

Figure 6. Chart showing some of the genes that are involved in regulating asymmetric cell divisions in neural stem cells in the development of the central nervous system of *Drosophilia*, where *Notch/Numb* are associated with the basal aspect of the cell, and genes such as *Inscuteable* are associated with the apical aspect. The related signaling pathways interact with cdc2 kinase and cyclins in the cell cycle. Similar networks of genes may be operating in mammalian stem cells during development and in the adult indicates suppression.

rare component of the tissue and hence carcinogen target cells would be likewise rare.

Probably the most definitive work involving stem cell markers for keratinocytes comes out of the laboratories of Morris in the U.S.A. and Kaur in Australia. The important feature of this work is that multiple criteria associated with stem cells (Li *et al*, 1998) have been studied simultaneously in primary cultures prepared from the basal layer of murine interfollicular epidermis (Tani *et al*, 2000). These cells with a long-term proliferative potential and a high short-term colony forming efficiency (stem or early lineage cells) strongly express the α_6 integrin (α_6^{bright}), and simultaneously weakly express a proliferation-associated cell surface marker initially recognized by a monoclonal antibody called 10G7 ($10G7^{dim}$) subsequently identified as the transferrin receptor (CD71). These early lineage cells could therefore be described as α_6^{bright} and $CD71^{dim}$, in comparison with transit amplifying cells, which are α_6^{bright} but also $CD71^{bright}$. In contrast postmitotic differentiated cells are α_6^{dim}. This work becomes more convincing when other marking approaches are combined with these criteria (Kaur and Li, 2000). Furthermore, tritiated thymidine LRCs (a putative way of marking stem cells by labeling template strands, see earlier) are predominantly (72%) α_6^{bright} $CD71^{dim}$. In contrast, the transit amplifying cells identified by pulse labeling with tritiated thymidine are predominantly α_6^{bright} $CD71^{bright}$, with only about 16% of these cells showing the pattern characteristic of stem cells (α_6^{bright} $CD71^{dim}$). Finally using cell size as a criterion for identifying the stem cells (small size or high nuclear to cytoplasmic ratio), it was again found that small size correlated with α_6^{bright} and $CD71^{dim}$. Although these studies were undertaken using fluorescence-activated cell sorting, it was also observed that $CD71^{dim}$ cells were also present *in situ* in the hair follicle bulb region. Such subtle surface protein changes in the keratinocyte hierarchy are reminiscent of those used to characterize hematopoietic stem cell populations ($CD34^{bright}$).

A recent excellent review by Watt and Hogan (2000) highlights one or two other important potential developments in terms of the molecular characterization of keratinocyte stem cells. These include the importance of the TCF/LEF family of transcription factors

Table I. Keratinocyte stem cells

Have a high colony forming ability *in vitro*. Holoclones.
Located in interfollicular epidermis, specific locations in a rete ridge configuration, in the bulb or matrix of hair follicles and in the bulge or upper outer root sheath.
Fixed cells (strong adherence or anchorage)
Can repopulate epidermis after culture
Long-term proliferative capability
Low proportion of cells in S + G2/M (long cell cycle)
Present in total keratinocyte population at 4–8^{+}%
Express p63 transcription factor[a]
Early lineage cells express $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins
Strong adhesion to basal lamina extracellular matrix, type IV collagen, or fibronectin
Express keratin 19
Express integrin α_6 strongly but weak expression of CD71[b] (α_6^{bright} CD71^{dim})
Retain ^{3}HTdR label when treated as babies (LRCs)
Small cells with a high nuclear to cytoplasmic ratio
The LRCs can form colonies *in vitro*
are predominantly α_6^{bright} CD71^{dim}
divide when skin is injured
tend to be at center of EPUs
retain radiolabeled skin carcinogens

[a]A member of the p53 family of transcription factors.
[b]A cell surface proliferation marker recognized by monoclonal antibody 10G7, which turned out to be transferrin receptor.

where knockout and mutant animals have apparent stem cell defects in the small intestine (TCF4) or hairs and whiskers (LEF 1) (Korinek *et al*, 1998). As β-catenin and WNT regulate this pathway these molecules may also be potential markers for early lineage cells.

There is clearly much further work required to identify the relationship between stem cells and the extracellular matrix to which they adhere. One feature of stem cells in renewing tissues such as epidermis and intestine is that these cells must somehow be protected against accidental exfoliation or migration and hence loss from the tissue. The implication is that they are bound more tightly or are fixed in some way at specific locations in the tissue (an argument against the idea that they may freely migrate through the tissue as has been proposed for the hair follicle bulge cells). The role of extracellular matrix proteins such as the integrins and integral membrane proteins such as the *trans*-membrane proteins of the *notch* family and its ligand *delta* are also worthy of further investigation (Lowell *et al*, 2000). Further studies are also needed to determine the factors (genes) that regulate stem cell divisions and control the possible asymmetric divisions assumed for steady state cell replacement. It is likely that the *notch* signaling pathways play an important role in conjunction with genes such as *numb*, together with genes such as *Musashi 1* and possibly *Miranda*, *Inscuteable*, etc., which play a role in developmental and invertebrate asymmetric stem cell systems (see **Fig 6**). It is clear that sonic hedgehog and its receptors Patched and Patched 2 as well as Wnt are important players in regulating hair growth and its morphogenesis (St-Jacques *et al*, 1998; Kishimoto *et al*, 2000; Wang *et al*, 2000; Botchkarev *et al*, 2001; Reddy *et al*, 2001; Sato *et al*, 2001; Yamago *et al*, 2001). These gene products inhibit the action of *serrate* in stimulating *Notch*. This raises the whole issue of the microenvironment surrounding stem cells (the niche) and the extent to which the niche is instructive or permissive in terms of stem cell function. This question is intimately linked with the issue of whether stem cells are intrinsically different from dividing transit cells, or whether they are merely instructed by their environment to behave as stem cells. The developing impression is that the cells are indeed intrinsically distinct although this does not decrease the importance of the microenvironment in terms of permissive instructions.

Human papilloma virus plays a role in the immortalization of keratinocytes *in vitro* and the activation of telomerase seems to play a key role in this process (Kang *et al*, 1998; Nakano *et al*, 1998; Steenbergen *et al*, 2001; Veldman *et al*, 2001). The precise role of telomere length and telomerase activity in the aging processes in skin, however, remains to be elucidated, particularly its role in relation to the stem cell population. It has been suggested, however, that telomerase levels in keratinocytes are correlated with adherence of cells in culture and with cells of colony forming ability and integrin β_1 staining (Yasumoto *et al*, 1996). This is somewhat contradicted by studies of telomerase activity in rapidly and slowing proliferating keratinocytes (Bickenbach *et al*, 1998) and in growing and resting hair follicles. Telomerase was high in growing follicle germs but low in catagen and telogen follicles (Ramirez *et al*, 1997), whereas Ogoshi *et al* (1998) noted hTER expression in both actively cycling and resting cells.

CONCLUSIONS

In conclusion, there have been significant advances in our understanding of keratinocyte stem cell biology since its early days in the 1970s, when the concept was initially proposed in relation to interfollicular epidermis and the EPUs. Subsequent important work drew attention to specific regions of the hair follicle outer root sheath where the skin's most potent reserve of stem cells resides. Unfortunately relatively little work has been directed towards characterizing in detail the proliferative activity and detailed migration pathways that occur in the germinal region of a growing hair follicle and as a consequence the identification of the location of lineage ancestor stem cells in this rapidly proliferating organ. The similarities between the hair follicle and the intestinal crypt, which is well understood (Potten *et al*, 1997; Potten, 1998; Marshman *et al*, 2002), are surprising and analogies can perhaps be drawn from one tissue to the other. There remains a major uncertainty about the precise location, numerical distribution, and characterization of the stem cells in the thicker regions of human epidermis where there are significant undulations in the basal layer associated with a rete ridge pattern. The studies to date, however, on isolated keratinocytes from mouse epidermis and *in situ* studies in interfollicular and follicular epidermis enable one to draw up a current list of the characteristics or markers associated with keratinocyte stem cells (**Table I**).

At present it would appear that the skin contains at least three distinct stem cell compartments. The interfollicular epidermis and the anagen hair follicle germinal matrix contain stem cells with a rather restricted differentiation potential, perhaps representing cells towards the end of the stem cell hierarchy shown as the upper circles in **Fig 1**. The simplest explanation at present is that in telogen the few hair follicle stem cells become quiescent awaiting the next hair growth cycle. The upper outer root sheath or bulge contains some potent reserve stem cells in adult skin. These cells can divide to repopulate many cutaneous structures including the epidermis, hair follicles, and sebaceous glands.

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